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[54]发明名称 骨生长刺激素注射剂及其制备方法

[57]摘要

本发明涉及一种从天然动物骨材料中提取的骨生长因子注射剂及其制备方法。含有牛骨形态发生蛋白 bBMP、成纤维细胞生长因子 bFGF 和聚乙烯吡咯啉酮 PVP；其制备过程为：脲素渗解，匀浆器匀浆，用透析膜透析 bBMP，同时加热溶解 PVP，再以此液溶解 bFGF；两液混合均匀，速冻，干燥，消毒，挥发后作需氧菌、厌氧菌培养，若培养呈阴性为成品。同现有技术相比，使用剂量小、次数少、对促进骨愈合及异位成骨效果明显。

权　利　要　求　书

1、一种骨生长刺激素注射剂，具有从天然动物骨材料中提取的骨生长因子，其特征在于：含有一种以上的外源性骨生长因子和致敏载体；外源性骨生长因子为牛骨形态发生蛋白（b BMP)或人骨形态发生蛋白(hBMP)和成纤维细胞生长因子(bFGF)或基因重组人成纤维细胞生长因子；致敏载体为聚乙稀吡咯啉酮(PVP)。

2、根据权利要求1所述的骨生长刺激素注射剂，其特征在于：外源性骨生长因子牛骨形态发生蛋白（b BMP)或人骨形态发生蛋白(hBMP)与致敏载体聚乙稀吡咯啉酮(PVP)的重量比为3:10；在此配比条件下需加入80000 IU单位的成纤维细胞生长因子(bFGF) 或基因重组人成纤维细胞生长因子。

3、一种根据权利要求1所述的骨生长刺激素注射剂的制备方法，其特征在于：

该配制品的制备过程为：

(1)、称取BMP1.5克，粉碎，用20毫升4M的脲素渗解，放置4℃冰箱中12小时后用匀浆器匀浆；

(2)、匀浆好的BMP用透析膜装好后打包，在4℃条件下，用4000毫升蒸馏水透析5～10次，每次间隔5～6小时；透析完全后，打开包再次匀浆，待用；

(3)、将5克PVP加入40毫升盐水中，加热溶解，置冷后过滤，并用2M的NaOH调PH至7.2，然后用此液稀释80000 IU单位的bFGF，待用；

(4)、将(2)液与(3)液混合均匀，用无菌盐水调至100ml，入百洁间，分装成瓶；置-75℃冰箱速冻5分钟，再置-30℃冰箱中冻20分钟，

送冻干机种冻干。

(5)、取出样品，置干燥塔内，并用环氧乙烷消毒； 然后再置百洁间中挥发，同时作需氧菌、厌氧菌培养；

(6)、若需氧、厌氧菌培养呈阴性，即为成品，压盖、 贴标签后置4℃冰箱保存。

说　　明　　书

骨生长刺激素注射剂及其制备方法

本发明属于医用配制品技术领域，涉及一种从天然动物骨材料中提取的骨生长因子注射剂及其制备方法。

在骨科医学界，传统的骨传导理论近年来受到骨诱导理论的冲击。用骨生长因子治疗骨折、骨缺损、骨不连等骨科疾病是国内外非常活跃的竞相开展的研究课题。大量的研究报告已经证实，骨形态发生蛋白BONE MORPHONETIC PROTEIN(以下简称BMP)可诱导血管周围游动间充质细胞转化为不可逆性的骨系胞，从而可在骨骼或骨骼以外部位产生软骨和骨组织，这一机理已被国内外骨科医学界所公认。在本发明以前的国内外现有技术中， 以BMP位基础的各项诱导成骨的研究工作开展的很多，例如：对骨形态发生蛋白(BMP)免疫原理与调节功能的研究；对内源性BMP及引导性骨再生的实验研究；对BMP与其它因子复和应用的研究；基因重组BMP的研究；载体对BMP活性影响的研究等等。已有实验证明，天然提取部分纯化的BMP有很好的骨诱导活性， 含有几种分子量的BMP较纯化单一分子量的骨诱导活性高，但成本高、 生产费时，不能满足需要。载体与BMP活性的关系是BMP的实验研究和临床应用中不可回避的问题，因为BMP在骨中的含量很少，提取量有限，使用高纯化的BMP又有被局部体液带走的危险。因此，BMP的应用必须同时考虑采用何种最佳载体的问题。现有技术中常用的载体有脱钙骨基质(DBM)、多孔磷酸钙(TCP)、羟基磷灰石(HAP)、 生物活性玻璃磁及纤维蛋白糊等，但这些载体都或多或少地存在这样或那样的问题。综上所述，虽然近年来， 以BMP为基础的骨诱导成骨的研究已形成高潮，有些工作也很有创新，但至今为止，属于很成熟的、能够用于临床的BMP医用配制品并不多见。 与上述骨诱导机理不同另一类的现有技术中，中国CN89105016.7号专利公开了一种”细胞生长刺激素及其

制造方法"。该药物中的主要成分为无菌血浆凝固酶，并含有蛋白质、多肽和多种氨基酸，是猪心肌蛋白胨和氯化钠水溶液作培氧基的谢金葡萄球菌代谢产物的提取液。这种促使骨折愈合及治疗溃疡的注射针剂，在治病机理上强调的是多种生物活性物质的协同作用，不需要考虑有效成分或有效部位作用机理和分离问题，因此，该药物在实际使用中的用量也非常大，需进行多次注射才显效果。目前，该药物已面市好几年，但至今对其药用机理和实际疗效没有明确的说法。与上述骨诱导理论指导下的骨生长因子治疗机理有着层次上的区别。

针对上述现有技术状况，本发明的目的在于：提供一种具有以天然提取部分纯化的BMP为基础的复合型骨生长因子和 具有有效缓释作用的最佳致敏载体的注射剂型医用配制品及制备方法。并且力求使其效果显著、需用量少、易于保存、使用方便。

现将本发明构思及技术解决方案叙述如下：

以BMP为基础的诱导成骨理论认为：诱导成骨必须具备三个条件，即：(1)诱导刺激物(如骨生长因子)；(2)间充质类细胞；(3)利于骨生长的血液供应环境。本发明在大量实验的基础上，对骨形态发生蛋白(BMP)与血管生成因子(FGF)的联合局部应用，作了深入的研究。实验结果证实，除了骨形态发生蛋白(BMP)可诱导血管周围游动的 间充质细胞转化为不可逆性的骨系胞，在骨骼或骨骼以外部位产生软骨和骨组织外，碱性成细胞纤维生长因子(FGF)，有刺激软骨细胞增质作用，是一种强大的毛细血管增殖刺激剂，而软骨内成骨和毛细血管的形成是骨诱导成骨后的重要步骤。实验还证明，聚乙稀吡咯啉酮(PVP)对蛋白质高分子物质具有良好的吸附作用，对骨生长因子——即骨形态发生蛋白和成纤维细胞生长因子的致敏作用十分明显，而且与二者的相溶性很好，可组成完全的混悬液，是一种有效的缓释载体。据此，本发明首先提供了一种骨生长刺激素医用配制品，其特征在于：含有一种以上的外源性骨生长因子和载体；外源性骨生长因子为牛骨形态

发生蛋白（b BMP)或人骨形态发生蛋白(hBMP)和成纤维细胞生长因子(bFGF)或基因重组人成纤维细胞生长因子；致敏载体为聚乙稀吡咯啉酮(PVP)；外源性骨生长因子牛骨形态发生蛋白（b BMP）或人骨形态发生蛋白(hBMP)与 致敏载体聚乙稀吡咯啉酮(PVP)的重量比为3:10；在此配比条件下需加入80000 IU单位的成纤维细胞生长因子(bFGF)或基因重组人成纤维细胞生长因子。其次，本发明还提供了骨生长刺激素注射剂的制备方法，其特征在于，该配制品的制备过程为：

(1)、称取BMP1.5克，粉碎，用20毫升4M的脲素渗解，放置4℃冰箱中12小时后用匀浆器匀浆；

(2)、匀浆好的BMP用透析膜装好后打包，在4℃条件下， 用4000毫升蒸馏水透析5～10次，每次间隔5～6小时；透析完全后， 打开包再次匀浆，待用；

(3)、将5克PVP加入40毫升盐水中，加热溶解，置冷后过滤， 并用2M的NaOH调PH至7,2，然后用此液稀释80000 IU单位的bFGF，待用；

(4)、将(2)液与(3)液混合均匀，用无菌盐水调至100ml，入百洁间，分装成瓶；置-75℃冰箱速冻5分钟，再置-30℃冰箱中冻20分钟，送冻干机中冻干。

(5)、取出样品，置干燥塔内，并用环氧乙烷消毒； 然后再置百洁间中挥发，同时作需氧菌、厌氧菌培养；

(6)、若需氧、厌氧菌培养呈阴性，即为成品，压盖、 贴标签后置4℃冰箱保存。

下面， 结合成纤维细胞生长因子(bFGF) 增强牛骨形态发生蛋白（b BMP)在小鼠体内的实验，将本发明医用配制品的骨生长刺激作用作进一步说明：

1、材料和方法：按文献报导的方法从新鲜小牛骨中提取部分纯化的bMP，经小鼠肌袋植入实验，证实其骨诱导活性。96mg的bMP溶于24

ml6M的脲素溶液，对水，4℃时透析24小时，得bBMP混悬液平均装入24个安瓿，冻干，封口备用。bFGF为冻干品。48只昆明小鼠，雄性，体重25g，随机分成4组。A组：用含bBMP100ng的PBS液（磷酸与磷酸盐的缓冲溶液）0.3ml注入含bBMP4mg的安瓿中，使成混悬液，吸入皮试注射器，注入小鼠股部肌肉内。B组：bBMP4mg/PBS液0.3ml混悬液注入小鼠股部肌肉内。C组：bFGF100mg/PBS液0.3ml。D组：PBS液0.3ml。均同法注射。21天时除死小鼠，切取注射部位的组织，用10% 中性福尔马林固定，盐酸脱钙24小时，水洗、脱钙、石蜡包埋，切片，HE染色，光镜下观察。各组其余标本用作钙含量测定。清除标本表面肌肉、匀浆、以10000rpm离心，沉淀物用盐酸消化，于原子吸收分光光度计测定钙含量，做为成骨定量的指标。

2、结果： A组小鼠肌注部位可触及一骨性硬结，组织学检查镜下可见有板层骨，骨小梁和红骨髓形成，间有纤维组织。 B组也有新骨形成，但未见新生毛细血管。C组和D组未见新骨。钙含量测定， A组是B组的3倍，是C组的8倍；B组大于C组和D组。

3、结论：含有多种骨诱导因子并与适合载体结合的医用配制品在骨科临床中具有广阔的前景。

本发明同现有技术相比，使用剂量小、次数少、对促进骨愈合及异位成骨效果明显，已可作为成熟的医用配制药品用于临床。

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PATENT-ASSIGNEE: WHOLE ARMY INST TRAUMATOLOGY & ORTHOPAED[WHOLN]

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BASIC-ABSTRACT:

NOVELTY - A bone-growth stimulant injection extracted from animal bones
contains ox-bone morphogenetic protein bBMP, fibroblast growth stimulant bFGF
and polyvinyl pyrrolidone PVP. It is prepared through such steps as dialysis
of urea, homogenizing, dialysis of bBMP, heating to dissolve PVP, dissolving
bFGF, mixing, quick freezing, drying, sterilization, volatilizing and culture
with aerobe or anaerobe to obtain finished product if it

shows negative. Its
advantages include low dosage, short course of treatment
and obvious effect on
promoting bone healing and malposition bone generation.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: OSSIFY INJECTION PREPARATION

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A BONE GROWTH STIMULANT INJECTION AND PREPARATION METHOD THEREOF
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TITLE (54): A BONE GROWTH STIMULANT INJECTION AND PREPARATION METHOD THEREOF

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[54] Name of Invention:

A Bone Growth Stimulant Injection And Preparation Method Thereof

[57]

This invention pertains to an injection of the bone growth factor extracted from natural animal bones and its preparation method. It contains bovine morphogenetic factor protein bBMP, fibroblast growth factor bFGF, and polyvinylpyrrolidone (PVP); its preparation method is as follows: bBMP is dissolved by liquid infiltration with urea, homogenized in a homogenizer and dialyzed with a dialysis membrane, and at the same time PVP is heated and dissolved, and bFGF is then dissolved in the same liquid; both liquids are mixed and rendered uniform, frozen, dried, sterilized and volatilized, whereupon they serve as a culture for aerobic bacteria and anaerobic bacteria; if the culture is negative, the product is ready. Compared to Prior Art, the dose used is small, the number of doses taken is small, and it has a pronounced effect in promoting bone healing and ectopic osteogenesis.

Scope of Patent Claims

1. A bone growth stimulant injection containing a bone growth factor extracted from natural animal bone material characterized in that it contains the above-mentioned exogenous bone growth factor and a sensitized carrier; the said exogenous bone growth factor is bovine bone morphogenetic growth protein or human bone morphogenetic growth protein (hBMP) and bovine fibroblastic growth factor bFGF or recombinant human fibroblast growth factor; the sensitized carrier is polyvinylpyrrolidone.

2. The bone growth stimulant injection recited in Claim 1 characterized in that the weight ratio of the bovine morphogenetic protein (bBMP) or human bone morphogenetic protein (hBMP), which is an exogenous bone growth factor, to the polyvinylpyrrolidone (PVP), which is the sensitized carrier, is 3:10; under this proportioning, 80,000 IU units of fibroblast growth factor (bFGF) or recombinant human fibroblast growth factor must be added.

3. A preparation method of the bone growth stimulant injection recited in Claim 1 characterized in that the preparation process of this formulation is:

(1) 1.5 g of BMP is weighed and taken, pulverized and dissolved by liquid infiltration with 20 mL of 4M urea, it is then allowed to stand for 12 hours in an ice box at 4 °C whereupon it is homogenized in a homogenizer;

(2) The homogenized BMP is packaged and dialyzed 5~10 times using an installed dialysis membrane with 4000 mL distilled water at 4°C at intervals of 5-6 hours; once the dialysis is finished, the package is opened, and its contents are ready for further use after another homogenization.

(3) 5 g PVP is added to 40 mL saline, heated and dissolved, cooled and strained, then its PH was adjusted to 7.2 with 2M NaOH, whereupon 80,000 units bFGF is diluted with this liquid, and ready for further use.

(4) Liquids (2) and (3) are mixed and homogenized, and adjusted to 100 mL with sterile saline, then [the product] is transferred to a clean facility for separate filling and packaging in bottles; it is then rapidly cooled in an ice box at -75°C for 5 minutes, left in an ice box at -30°C for another 20 minutes and transferred to a freeze drier to freeze-dry.

(5) Samples are taken, placed in a drying tower, and sterilized with epoxy ethane, whereupon they are placed again in a clean facility to volatilize and at the same time serve as a culture for aerobic and anaerobic bacteria.

(6) If the aerobic and anaerobic bacteria cultures display negativity, the product is ready; it is then closed, labeled, and placed for storage in an icebox at 4°C.

Specification

A Bone Growth Stimulant Injection And Preparation Method Thereof

This invention pertains to an injection of the bone growth factor extracted from natural animal bones and its preparation method.

Lately in traumatology and orthopedics, the bone conduction theory has come under attack from the bone induction theory. The treatment of bone fractures, defects, non-unions and other orthopedic conditions with the bone growth factor is a dynamic, competitively developing field of studies both in China and abroad. Numerous papers have already proven that bone morphogenetic protein (hereinafter abbreviated to BMP) can induce perivascular migrating mesenchymal cells to transform into irreversible bone cells and thus generate cartilage and bone tissue within the skeleton and outside the skeleton. This mechanism has been acknowledged both in the orthopedic circles both in China and abroad. In both domestic and foreign Prior Art, plenty of research has been carried out based on induced bone formation based on BMP, such as a study of the immunological principles and regulatory functions of bone morphogenetic protein (BMP), an experimental study of endogenous BMP and guided bone regeneration; a study on the combined use of BMP with other factors; a study on the use of recombinant BMP; a study on the impact of the carrier on BMP activity, etc. Experiments conducted so far have proved that naturally extracted and partially purified BMP

has a very good bone induction activity, and that the BMP that includes several molecular weights has a higher bone induction activity than the purified BMP with a single molecular weight; however, it cannot satisfy the demand because of the high cost and time-consuming production. The relation between the carrier and BMP activity is an inevitable problem of experimental research and clinical applications since the quantity of BMP in the bones is very small, its extracted amount is limited, and when high-purity BMP is used there is also the risk that it will be carried away by the local body fluids. Therefore in BMP applications, the issue of which carrier is optimal must be given consideration. Carriers that have been commonly used in Prior Art include decalcified bone matter (DBM), porous tricalcium phosphate (TCP), hydroxyapatite (HAP), bioactive glass ceramics, etc; however, all these carriers have some problems or other to a certain extent. To summarize the above, though recent years have seen an upsurge of research on bone formation by bone induction based on BMP, and some of the studies are quite innovative, so far, there are very few medical preparations of BMP that are fully mature and suitable for clinical use. In a technology different from the above bone induction mechanism, the Chinese Patent CN89105016.7 discloses "a cell growth factor and preparation method thereof." The main component in this medication is sterile plasma-coagulase; it also contains protein, polypeptides, and various amino acids. This is an extract staphylococcus metabolite cultivated on pig myocardium

peptone and an aqueous solution of sodium chloride. This kind of injection preparation for the healing of bone fractures and ulcers places an emphasis on the synergistic effect of various bioactive substances as its therapeutic principle, without a need to consider and separate the action mechanisms of effective ingredients or the effect locations. Therefore in practice such medications are administered in extremely large amounts; it takes multiple injections to produce an evident effect. This medication has been on the market for a few years now, but to this day there is no way to determine for sure its medicinal mechanism and whether or not it has a practical effect. Its therapeutic mechanism is on a different level than that of the bone growth factor under the above-mentioned bone-induction theory.

In view of the above-mentioned situation of Prior Art, the purpose of the present invention is to offer a medicinal preparation in the form of injections that has a complex form of bone growth factor based on naturally extracted and partially purified BMP and the optimal sensitized carrier with an effective slow release action, as well as a preparation method thereof. We also strived to make its effect remarkable, required amount small, make it easy to store and convenient to use.

We will now describe the concept and technical approach of this invention as follows.

BMP-based induced bone formation theory claims that three conditions must be satisfied to induce bone formation: (1) the presence of the inducing stimulant (such as bone growth factor); (2) mesenchymal cells; (3) delivery of blood that promotes bone growth to the environment. This invention is based on a vast experimental foundation; we conducted an in-depth study of the combined local application of bone morphogenetic protein (BMP) and blood vessel growth factor (FGF). The results of the experiments demonstrated that in addition to bone morphogenetic protein (BMP) being able to induce perivascular migrating mesenchymal cells to transform into irreversible bone cells and thus generate cartilage and bone tissue in locations within the skeleton and outside the skeleton, the alkaline fibroblast growth factor (FGF) also has the effect of stimulating the proliferation of cartilage cells; it is a powerful stimulant of capillary proliferation, and bone formation and capillary formation inside cartilage are major steps in the bone formation by bone induction. The experiments proved, moreover, that polyvinypyrrolidone (PVP) has an excellent adsorption effect on the macromolecular compounds of proteins, having an absolutely clear sensitizing effect on the bone growth factor, i.e., bone morphogenetic protein, and the fibroblast growth factor. It also possesses good mutual solubility with both factors, such that a complete suspension can be prepared, which is an effective slow release carrier. Based on this, this invention offers, first of all,

a medicinal preparation characterized in that it contains one or more exogenous bone growth factors and a carrier; the exogenous bone growth factor is bovine bone morphogenetic protein (bBMP) or human bone morphogenetic protein (hBMP) and fibroblast growth factor (bFGF) or recombinant human fibroblast growth factor; the sensitized carrier is polyvinylpyrrolidone. The weight ratio of the bovine bone morphogenetic protein (bBMP) or human bone morphogenetic protein (hBMP), which is the exogenous bone growth factor, to the polyvinylpyrrolidone (PVP), which is the sensitized carrier, is 3:10; under this proportioning, 80,000 IU units of fibroblast growth factor (bFGF) or recombinant human fibroblast growth factor must be added. Furthermore, this invention also offers a preparation method of the bone growth stimulant injection characterized in that characterized in that the preparation process of this formulation is:

(1) 1.5 g of BMP is weighed and taken, pulverized and dissolved by liquid infiltration with 20 mL of 4M urea, it is then allowed to stand for 12 hours in an ice box at 4 °C whereupon it is homogenized in a homogenizer;

(2) The homogenized BMP is packaged and dialyzed 5~10 times using an installed dialysis membrane with 4000 mL distilled water at 4°C at intervals of 5-6 hours; once the dialysis is finished, the package is opened, and its contents are ready for further use after another homogenization.

(3) 5 g PVP is added to 40 mL saline, heated and dissolved, cooled and strained, then its PH was adjusted to 7.2 with 2M NaOH, whereupon 80,000 units bFGF is diluted with this liquid, and ready for further use.

(4) Liquids (2) and (3) are mixed and homogenized, and adjusted to 100 mL with sterile saline, then [the product] is transferred to a clean facility for separate filling and packaging in bottles; it is then rapidly cooled in an ice box at -75°C for 5 minutes, left in an ice box at -30°C for another 20 minutes and transferred to a freeze drier to freeze-dry.

(5) Samples are taken, placed in a drying tower, and sterilized with epoxy ethane, whereupon they are placed again in a clean facility to volatilize and at the same time serve as a culture for aerobic and anaerobic bacteria.

(6) If the aerobic and anaerobic bacteria cultures display negativity, the product is ready; it is then closed, labeled, and placed for storage in an icebox at 4°C.

Below is a summary of in vivo experiments on mice using bovine bone morphogenetic protein (bBMP) strengthened with fibroblast growth factor (bFGF), and we will further explain the bone growth stimulating effect of the medicinal preparation of this invention.

1. **Materials and method.** Using the method described in literature, we extracted partially purified newborn calf's bBMP and

experimented by implanting it in a mouse muscle pouch, proving its bone induction activity. 96 mg bBMP was dissolved in 24 mL of 16 M urea solution; it was dialyzed with regard to water for 24 hours, and the obtained bBMP suspension was evenly distributed into 24 ampoules, freeze-dried and sealed for further use. bFGF was a freeze-dried product. 48 male Kunming mice, with a body mass of 25 g each were randomly separated into 4 groups. Group A: 0.3 ml of liquid PBS (a buffer solution of phosphoric acid and its salts) containing 100 mg bBMP was injected into ampoules containing 4 mg bBMP to prepare a suspension, which was injected into the musculus gracilis of the mice using a skin-suction type injector. Group B: 0.3 mL of bBMP 4 mg/liquid PBS suspension was injected into the musculus gracilis of the mice. Group C: 0.3 mL bFGF 100 mg/liquid PBS. Group D: 0.3 mL liquid PBS. In 24 days, the mice were put to sleep, and the tissues in the injected part were excised and fixed in 10% neutral formalin. The tissues were decalcified with hydrochloric acid for 24 hours and then rinsed; the calcium was removed. The tissues were then embedded in paraffin, sliced, HE stained, and studied by means of optics. Other specimens from each group were used to determine calcium content. The specimens were cleaned on the surface, homogenized and centrifuged at 1000 rpm, the deposit matter was digested with hydrochloric acid, and the calcium content of the deposit matter was determined by atomic absorption spectrophotometry to serve as the quantitative index of bone formation.

2. **Results:** In the injected location of group A mice bony hardening could be felt by touch. Under histological examination lens, lamellar bone was observed, as well as trabeculation and red bone marrow formation, with fibrous tissues in the space. Group B also revealed new bone formation, but not the formation of new capillaries. Groups C and D revealed no new bone. By the determined calcium content, group A exceeded group B by a factor of 3, and group C by a factor of 8; group B exceeded both group C and group D.

3. **Conclusion:** The medicinal preparation that contains several kinds of bone induction factors and is combined with a suitable carrier has broad prospects in the orthopedic clinical practice.

Compared to Prior Art this invention uses a smaller dose, fewer applications, has a clear effect of promoting bone healing and ectopic bone formation; it can be used as a mature medicinal preparation in clinical practice.